# Peptide-mediated cell delivery: application in protein target validation

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Recent reports have suggested that conjugation of peptides, proteins and antisense to short highly basic peptides, such as TAT, antennapedia and transportan, results in their rapid translocation into cells. Importantly, these conjugates have been shown to exert actions in a number of animal models suggesting their general utility for the determination of protein function *in vitro* and *in vivo*.

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**Abbreviations**
PNA peptide nucleic acid
PTD protein transduction domain

## Introduction

The complexity of biological interactions makes it increasingly difficult to predict gene and protein function as you proceed from the immediate metabolic pathway to the cellular and animal level. This phenomenon is partially responsible for the high attrition rate observed during drug development and necessitates early validation of protein function in cellular and/or animal models that are predicative of the disease. Classically, this is undertaken either through acute modulation using protein knockdown and/or inhibition, or using chronic models, such as transgenic and knockout mice.

In cellular systems, a variety of tools are employed to determine protein function, including antisense, peptide modulators and the overexpression of wild-type or dominant-negative protein. However, these studies are often limited by the inability to effectively deliver these validation tools. Typically, delivery is via lipids, electroporation or through viral vectors, but these have several severe limitations, including the inability to deliver to primary, non-dividing cells, the requirement for optimisation with each cell type, low transfection levels and cellular toxicity. Interestingly, recent studies have identified several short peptide sequences named protein transduction domains (PTDs) or cell penetrating peptides (CPPs), which appear to rapidly translocate into all cells both *in vitro* and *in vivo*. Importantly, conjugation of proteins, peptides and antisense to these PTDs has been shown to deliver these cargos effectively, allowing observation of biological action in several cell and animal models [1,2]. In this review, we examine the use of PTDs as a novel and potentially universal delivery system for delineation of protein function and target validation.

## Peptide transduction domains

PTDs were first identified while investigating the spontaneous cell entry of HIV TAT, which is subsequently localised to the nucleus and transactivates the viral long-terminal repeat promotor encoded within the human immunodefficiency virus [3]. Studies of the minimum translocation region identified a positively charged section between amino acids 47 and 57, which was previously associated with DNA binding [4]. Similar studies of antennapedia, a *Drosophila* homeodomain transcription factor, identified a 16-amino-acid PTD derived from region 43–58, and also located within the DNA-binding third domain [5–7] Since these initial observations, a host of short peptides have been identified and shown to rapidly translocate across membranes. However, conjugation and delivery of biological cargos has been predominantly performed using the peptides derived from TAT (sequence, in single-letter amino acid code: YGRKKRRQRRR), antennapedia (RQIKIWFQNRRMKWKK) and transportan (GWTLNSAGYLLGPHIDNHRSFHDKYGLA), a synthetic chimera derived from galanin and mastoparan [8].

## Mechanism of cell entry

To date, studies of the mechanism of cell entry have largely examined the movement of labelled antennapedia and TAT across artificial membrane systems or into cells. Although this pathway is probably analogous to the situation during PTD-mediated delivery of small peptide cargos, it is likely that the mechanism will be different with larger macromolecules such as antisense and proteins. With this caveat in mind, the studies have identified several important characteristics, outlined below.

### Dependence on basic arginine residues

Substitution of alanine for arginine in the TAT sequence significantly attenuated translocation, whereas simple polyarginine sequences, with an optimal length of 8–9 mer, produced uptake rates that were significantly higher than TAT [9,10]. The guanidine headgroup is the critical structural feature, as homopolymers of citrulline, an arginine isostere, show no transduction, whereas increasing the distance between the guanidine group and the peptide backbone, using alkyl spacers, further increased uptake [11].

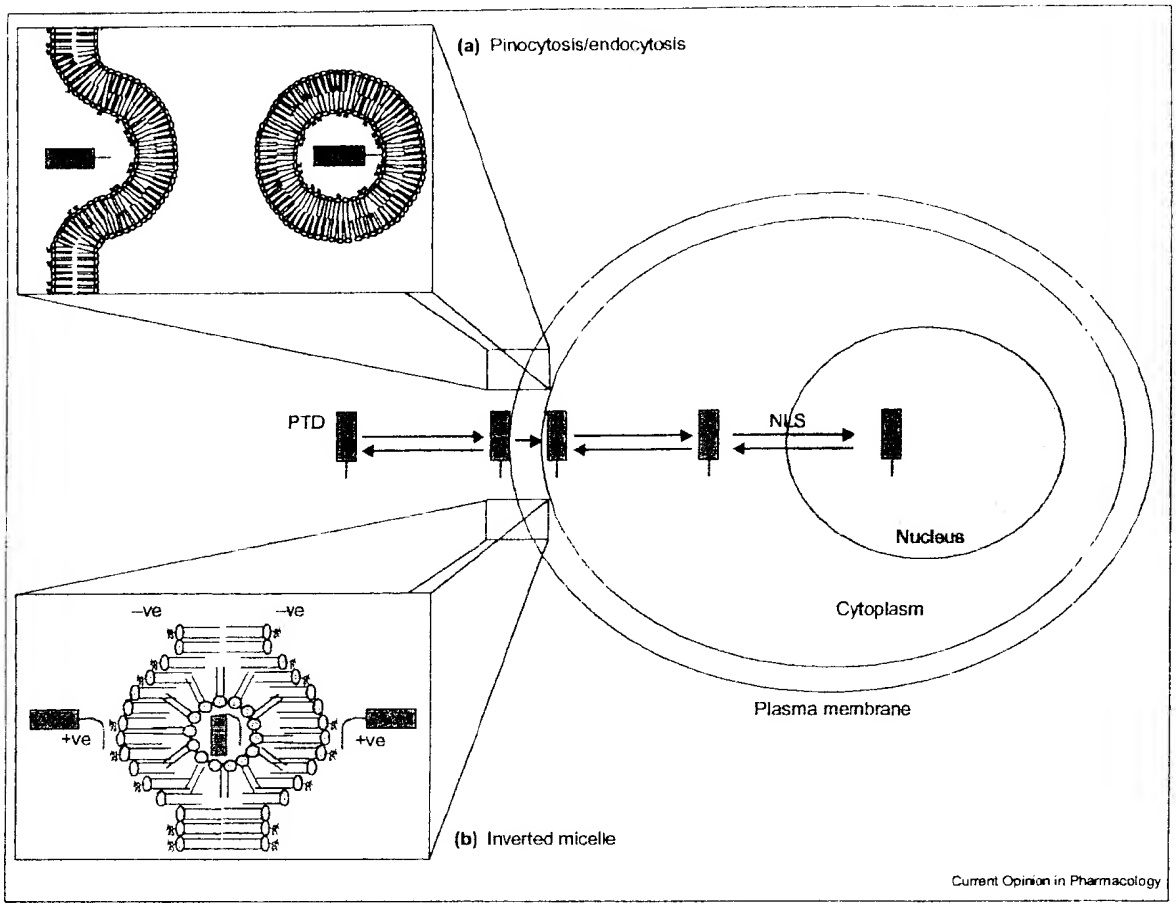
### Independence of the endocytic pathway

Uptake is unaffected by a range of endocytosis inhibitors, including wortmannin, cytochalasin D, colchicines, nocodazole, taxol, brefeldin A and chloroquine, and is only partially attenuated when the temperature is reduced from 37°C to 4°C [4,5,12].

### Energy-independent mechanism

Uptake is unaffected by metabolic inhibitors, including sodium azide and rotenone [4,5,12].

**Figure 1**




Mechanism of PTD-mediated delivery. Initial binding is mediated through electrostatic interaction between positively charged (+ve) PTD sequences (purple line) and biological cargo (red box) and negatively charged (–ve) membrane phospholipids (grey circles). Little is known of the subsequent cellular uptake mechanism, although delivery by both **(a)** an as yet undefined pinocytosis/endocytosis pathway and **(b)** inverted-micelle-mediated membrane shuttling produced by the interaction of hydrophobic amino acids and the membrane has been proposed. Once released into the cytoplasm, PTD-conjugates can be targeted to the nucleus using nuclear translocation sequences (NLS).

### Receptor-independent mechanism

Delivery of antennapedia [5,13] and TAT [10,11] is unaffected by the retro-inverso sequence or when these PTDs are composed entirely of D-enantiomers, indicating that the process is not dependent on receptors.

The identification of micelles during nuclear magnetic resonance studies of antennapedia translocation across phospholipid membranes [14], prompted Alain Prochiantz to propose an inverted micelle model to explain antennapedia translocation (Figure 1). This envisages initial electrostatic interaction between the peptide (positive charge) and membrane phospholipid (negative charge) followed by plasma membrane shuttling, through formation of unilamellar phospholipid bubbles. However, inverted micelle formation requires the presence of hydrophobic amino acids that are absent in TAT and other PTDs, implying that this is unlikely to represent the common PTD delivery mechanism. Alternatively, although there are no studies to date that have addressed this issue, it would appear more likely that PTD delivery of proteins and antisense is mediated via an as yet uncharacterised pinocytosis/endocytosis-related mechanism (Figure 1).

Central to using PTDs is not only their ability to deliver tools but, importantly, to deliver tools at concentrations and for time periods sufficient to give biological activity. Comparison of the uptake rate by fluorescence resonance energy transfer (FRET) analysis showed that the transportan uptake rate was greater then TAT, which was greater than antennapedia and, like many other studies using labelled PTD, that uptake was essentially complete within 15–60 min [15•]. Examination of intracellular PTD levels showed that these are correlated with the rate of

**Table 1**

**PTD-mediated peptide delivery.**

| Target | PTD | Cell type | Cellular response | Ref |
|---|---|---|---|---|
| IL-4 signalling (SH2 domain) | Ant | RAMOS, Caki-1, PBL and HeLa | Inhibition of Stat6 phosphorylation | [35] |
| $\beta_3$ integrin | TAT | Neutrophils | Inhibition of PI3K and transmatrix migration | [36] |
| JNK-1/2 | TAT | TC3 | IL-1β induced JNK activation and apoptosis | [37] |
| p53-mdm2 binding | Ant | Cancer cell lines including BMRPA1, E49 and HeLa | Induced apoptosis | [38] |
| BAD (BH3 domain) | Ant | HeLa, CEM and 32D cells | Apoptosis induction | [39] |
| NADPH oxidase | TAT | *In vivo* (rat) | Inhibition of angiotensin-induced $O_2^-$ levels in aortic rings and systolic blood pressure | [33] |
| IKK2 | Ant | HeLa and *in vivo* (mouse) | Inhibition of NEMO (IKK3) induced IKK2 activation *in vitro* and acute inflammatory response *in vivo* | [34] |
| G1 cyclin–Cdk complex | Ant | AsPC-1 and BxPC-3 | $p16^{INK4A}$-derived peptide attenuated cell proliferation | [40] |
| cGMP-dependent protein kinase 1α | TAT<br>Ant | *In vivo* (rat) | NO-induced cerebral dilutation in rat cerebral artenes | [41] |
| PDGF/IGF/insulin signalling (SH3 domain) | Ant | NIH3T3 | DNA synthesis and proliferation | [42] |
| PKC RACK binding | Ant | *Ex vivo* (rabbit adult ventricular myocytes) | Abolished the protection derived from ischaemic pre-conditioning | [43] |
| Grb2-Sos binding during NGF/EGF signalling (SH3 domain) | Ant | ER22/PC12/ NIH3T3/HER2 | Grb2–Sos binding, ERK-1/2 activation and proliferation | [44] |
| Bak (BH3 domain) | Ant | HeLa cells | Inhibited apoptosis | [45] |
| Cdk2/4 | Ant | CA46 | $p21^{Waf1/Cip1}$-derived peptide attenuated cell growth | [46] |
| Inhibition of GRB10-receptor binding (SH2/SH3 domain) | Ant | NIH3T3 cells | DNA synthesis and proliferation | [47] |
| PI3K (SH2 domain) | Ant | Cerebellar granule cells | Apoptosis | [48] |
| c-Myc-DNA binding | Ant | MCF-7 | Cell growth/apoptosis and ODC/p53 regulation | [49] |
| Cdk4/6 | Ant | HaCaT cells | $p16^{INK4A}$-derived peptide attenuated cell growth | [18] |
| CD44 cytoplasmic domain | Ant | RPM-MC | Cell migration | [50] |
| Phosphatidylinositol 3-kinase (SH2 domain) | Ant | C2 muscle cell | Stimulates entry into S-phase | [51] |
| Cdk2/4 | Ant | SKOV-3/IGROV-1 | $p21^{Waf1/Cip1}$-derived peptide attenuated Cdk activity and cell proliferation | [52] |
| EGF/PDGF signalling (SH2 domain) | Ant | Newt A1 myoblasts and mammalian C2 myoblast cells | Proliferation and ERK-1/2 activation | [53] |
| FGF signalling (SH2 domain) | Ant | Rat small cerebellar neurons | PLCγ activation and neurite outgrowth | [19] |
| EGF signalling (SH2 domain) | Ant | SAA cells | Ras and ERK-1/2 activation | [54] |

Ant, Antennapedia; CdK, cyclin-dependent kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; JNK, c-Jun N-terminal kinase; IGF, insulin-like growth factor; IKK, IκB kinase; IL, interleukin; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; RACK, receptor for activated C kinase.

uptake and, interestingly, that these are significantly higher than those in the extracellular media. Thus, incubation for 60 min with 5 μM labelled peptide gave intracellular concentrations of 1800 μM, 500 μM and 350 μM for transportan, TAT and antennapedia, respectively [15•], an observation confirmed by high-performance liquid chromatography determination of antennapedia delivery [16]. Interestingly, it also appears that the uptake level is comparable across different cell types and is independent of the PTD, implying that they employ similar delivery mechanisms [12,16].

## Peptide delivery

Both antennapedia and TAT have been used in peptide delivery and have been effectively employed to attenuate protein–protein and enzyme–substrate interactions involved in growth factor, cytokine and integrin signalling, apoptosis, and cell division (Table 1; [17••]). Examination of the literature shows that, compared with PTD–protein conjugates, substantially greater concentrations of PTD–peptide conjugates (>10 μM) were required for biological activity. The proteins are likely to have a greater affinity, resulting from their secondary and tertiary structure. This was clearly shown in a study by Fahraeus *et al.* [18], who found that the full-length protein and a short inhibitory peptide sequence derived from p16 INK4A, gave $IC_{50}$s of 10 nM and 10 μM, respectively, when measuring inhibition of the kinase cdk4/6. It thus appears that the most suitable peptide modulators for this approach would be short in length and require no secondary and tertiary structure for specificity and binding. As an example, PTD-mediated delivery of phosphotyrosines has been successfully employed to attenuate binding through SH2 domains. This inhibition is often very selective because

**Table 2**

**PTD-mediated protein delivery.**

| Protein | PTD | Cell type | Cellular response | Ref |
|---|---|---|---|---|
| APO-BEC-1 | TAT | Primary hepatocytes | Induced apoB mRNA editing and enhanced synthesis/secretion of VLDL | [55] |
| Cre recombinase | TAT | CV-1 fibroblasts, mouse embryonic stem cells and splenocytes | Cre recombination of loxP sites | [32] |
| Catalase | TAT | PC12 and mouse skin | Cell viability following oxidative stress | [24] |
| RhoA | TAT | Human eosinophils | Migration | [56] |
| H-Ras | TAT | Human eosinophils | IL-5 induced ERK-1/2 activation, p90 Rsk1 phosphorylation and survival | [57] |
| Filamin-1 | TAT | HEK-293 | ERK-1/2 activation | [58] |
| IκBα | TAT | Mouse bone marrow macrophages and osteoclasts | Osteoclastogenesis | [59] |
| β-Galactosidase/ Bcl-$X_L$/PEA-15 | TAT | Rat pancreatic islets (β-cells) | Apoptosis | [60] |
| p16 | TAT | Primary human diploid fibroblasts | $G_1$ cyclin–Cdk complexes | [61] |
| HPC-1/syntaxin | TAT | PC12 | $K^+$ and carbachol induced noradrenaline and dopamine release | [62] |
| Cdk2 | TAT | Human mammary epithelial cells | Cell division progression via the $\alpha_2\beta_1$integrin | [63] |
| Rho/Rac/Cdc42 | TAT | Human dermal microvascular endothelial cells | VEGF-stimulated endothelial cell motility | [64] |
| RhoA | TAT | Avian osteoclast | PI3K activation, podosome assembly, stress fibre formation, osteoclast motility and bone resorption | [65] |
| Superoxide dismutase | TAT | HeLa | Oxidative stress | [23] |
| E2F-1/p73/p53 | TAT | Jurkat T-cells/primary T-cells | TCR activation induced cell death | [66] |
| Caspase-3 | TAT | Jurkat T-cells | HIV-protease activation in infected cells cleaved and activated caspase-3 to induce cellular apoptosis | [67] |
| $p16^{INK4A}$ | Ant | Primary human diploid fibroblasts | Growth arrest | [20] |
| $p27^{Kip1}$ Cdk inhibitor | TAT | Jurkat T-cells, hepatocellular carcinoma | Cell division and migration | [68] |
| $p16^{INK4A}$ | TAT | HaCaT/HepG2/Jurkat T-cells | Retinoblastoma phosphorylation | [69] |

Cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IκB, inhibitor of NFκB; IL, interleukin; PI3K, phosphatidylinositol-3-kinase; TCR, T-cell receptor; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein.

the specificity of SH2 binding to a particular phosphotyrosine sequence is determined by the three to five amino acids that are C-terminal to the tyrosine. Thus, a peptide based around a phosphotyrosine site (Tyr766) upon the fibroblast growth factor receptor 1 (FGFR-1) and attached to antennapedia selectively inhibited phosphatidylinositol signalling and subsequent neurite outgrowth in primary neurons, but had no effect upon survival, differentiation or phosphatidylinositol hydrolysis stimulated by platelet-derived growth factor, neurotrophin-3 or bradykinin [19].

## Protein delivery

Although antennapedia-mediated [20] and transportan-mediated [21] protein delivery has been reported, Steven Dowdy has pioneered this area using TAT-conjugated proteins [22•]. As with the peptides, this has been successfully employed to deliver several targets, including dominant-negative GTP-binding proteins (Rho, Rac, Cdc42), modulators of cyclin-dependent kinase (cdk) activation ($p16^{INK4A}$ and $p27^{KIP1}$) and inhibitor of NFκB (IκB)-α (Table 2). Furthermore, as discussed earlier, TAT proteins appear to be considerably more efficacious than peptides, often giving biological activity at submicromolar concentrations.

Few investigators have examined the mechanism of TAT-mediated protein uptake, although recent observations imply that this may differ significantly from that of TAT-mediated peptide uptake. Measurement of catalase and superoxide dismutase (SOD) delivery by TAT, determined by western blotting, showed uptake at 10–30 min, which peaked at 4–6 hours before dropping to basal levels at 48–72 hours [23,24]. However, in contrast to TAT–peptide uptake, TAT–protein uptake (of catalase and SOD) was found to be attenuated at 4°C [25•] and to be dependent on binding to the negatively charged heparin sulfate proteoglycans [12,25•,26]. With a few exceptions, it is also thought that TAT–protein uptake is facilitated when proteins are in a partially re-natured state. For example, SOD and catalase uptake fails to occur when these proteins are prepared in the native state [23,24]. The reason for this phenomenon is unknown, although it has been speculated that the uptake mechanism is driven by the decrease in entropy following protein refolding within the cell. Alternatively, it might also be envisaged that the partially re-natured state might permit more efficient interaction of TAT with the positively charged cellular membrane.

**Table 3**

**PTD-mediated antisense delivery.**

| Target | PTD-conjugate | Cell type | Cellular response | Ref |
|---|---|---|---|---|
| PTPσ | Transportan-PNA | Rat islets of Langerhans | Inhibited glucose-induced insulin secretion | [27] |
| c-myc antigene | Nuclear localisation sequence-PNA | BJAB/HBL2 | Inhibited cell cycle and apoptosis | [28] |
| P-glycoprotein | Ant-ODN, TAT-ODN | NIH3T3 | P-glycoprotein expression | [70] |
| Galanin receptor | Ant-PNA, transportan-PNA | Bowes/rat (*in vivo*) | Decreased galanin expression and inhibited C-fibre stimulation-induced facilitation of the rat flexor reflex | [29] |
| Amyloid precursor protein | Ant-ODN | Rat cortical neurones | Axon and dendrite outgrowth | [71] |

ODN, oligodeoxyribonucleotide; PTPσ, protein tyrosine phosphatase σ.

## Antisense delivery

Although a limited number of studies have employed TAT, transportan and antennapedia for delivery of antisense, their general utility has been limited by the difficulty in chemically conjugating the PTD peptide and antisense oligonucleotide backbones (Table 3). However, this problem can be resolved through the use of peptide nucleic acid (PNA) antisense, which has been shown to attenuate protein expression and biological activity of a small number of targets, including protein tyrosine phosphatase σ [27], c-myc [28] and the galanin receptor [29]. Interestingly, the attenuation of c-myc expression was through an action of PNA at the transcriptional level [28]. With few publications in this area it is difficult to draw lessons regarding the mechanism or utility of PTD–antisense delivery. However, it could potentially provide an extremely useful and general tool because, unlike peptides and proteins, it should be possible to examine the role of unknown genes.

## *In vivo* delivery

To date, only a small number of studies have investigated the *in vivo* utility of PTD-mediated protein delivery. Considerable interest was first generated by a report demonstrating the presence of active TAT–β-galactosidase in all mice tissues (including the brain) at 4–8 h following intraperitoneal injection [30••]. Since then, a study by Jo *et al.* [31••] has demonstrated Cre delivery to both mammalian cells and mouse tissue, using a PTD derived from Kaposi fibroblast growth factor (FGF)-4 [31••]. Paradoxically, a comparable investigation showed that TAT, but not FGF, mediated cellular Cre delivery when combined with a nuclear localisation sequence [32]. Overall, these two studies suggest that PTD-mediated Cre delivery could be of enormous potential for the conditional knockout of loxP-flanked genes both *in vitro* and *in vivo*. Interestingly, a recent study had shown that following topical application of TAT–catalase to mouse skin, activity was found to penetrate the epidermis as well as the dermis of the subcutaneous layer [24] suggesting that it may be possible to selectively target PTD cargos.

Like PTD-mediated protein delivery, only a few investigations have tackled the use of PTD-mediated peptide delivery, and these have focused on delivery to inhibit *in vivo* function. Thus, peptide inhibitors conjugated to PTD designed to attenuate the activation of NADPH oxidase and IκB kinase 2 (IKK2) have been shown to inhibit rat-angiotensin-induced increases in systolic blood pressure in mice [33] and acute mouse inflammatory responses [34], respectively. Similarly, a single study has examined PTD–antisense delivery and showed that transportan-mediated PNA antisense delivery caused downregulation of galanin receptor expression and inhibition of C-fibre stimulation-induced facilitation of the rat flexor reflex [29].

## Conclusions

The recent completion of the first draft of the human genome has provided the scientific community with the majority of the basic building blocks responsible for human biology. The next phase, involving the identification of gene function, would be greatly facilitated by the ability to deliver tools that modulate protein function *in vitro* and *in vivo*. To this end, initial studies have suggested that PTDs may provide a valuable, possibly universal delivery tool for the acute *in vitro* and *in vivo* delivery of peptides, proteins and antisense. However, several questions will need to be addressed to define the overall applicability of this technology. Most importantly, although it appears that initial binding is mediated following electrostatic interaction at the cell membrane, little is known of the mechanism of cellular uptake, trafficking and metabolism, the differences between PTDs and how these are affected following conjugation to proteins and antisense. Hopefully, an understanding of these factors will permit the optimal design of PTD cargos for the intracellular targeting of the protein of interest.

## Update

Continuing studies on the mechanism of PTD-mediated uptake have confirmed the importance of positively charged amino acids and demonstrated highly efficient delivery of proteins >500 kDa following conjugation to polylysine sequences [72]. Interestingly, a recent study using confocal microscopy identified the presence of labelled PTD–PNA conjugates in cytosolic vesicular compartments and suggested that delivery is mediated via a receptor-dependent endocytotic pathway [73••]. In both publications, uptake was found to be cell-type-dependent [72,73••].

Of particular interest is a recent *in vivo* study in mice, using a TAT–BcL-xL construct that was found to cross the blood brain barrier and transduce into neurons of various brain regions within 1–2 h of intraperitoneal injection [73••]. Importantly, using a murine model of focal ischemia/reperfusion, treatment with TAT–BcL-xL was found to decrease cerebral infarction through inhibition of caspase-3 activation and neuronal apoptosis [73••].

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This paper demonstrates, for the first time, the utility of a peptide–protein conjugate for investigating the role of a protein target in an *in vivo* disease model.